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The work in this report deals with the isolation and purification of ciguatoxin from ciguateric fish, the pharmacology and toxicology of ciguatoxin and other toxins extracted from various assays and progress made on a system for detection of ciguateric fish flesh. Ciguatoxin has been isolated from livers and viscera of eels obtained from Pacific Islands where ciguatera is endemic. Evaluation of the pharmacology and toxicology has been both in vitro and in vivo systems but has been hampered by a shortage of purified toxins. The immunological "stick method" of assaying for ciguatera in fish has yielded interesting and promising results. Work on all of these aspects of ciguatera toxicity and toxicity to other marine toxins is proceeding at an accelerated pace.

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# A MULTIDISCIPLINARY STUDY OF CIGUATOXIN AND RELATED LOW MOLECULAR WEIGHT TOXINS FROM MARINE SOURCES

ANNUAL AND FINAL REPORT

LESLIE E. BAILEY
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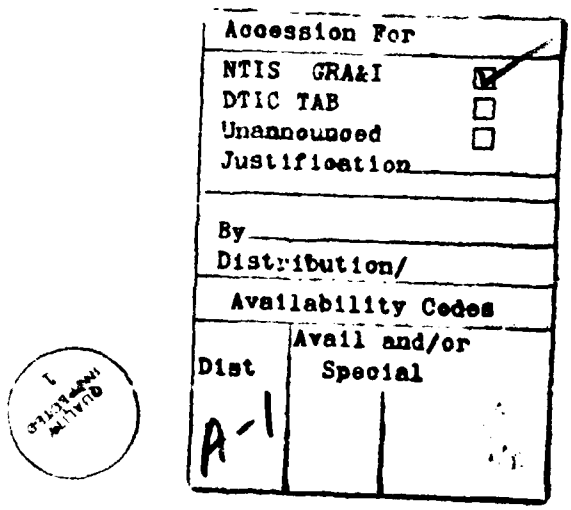

## FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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Annual and Final Report

L.E. Bailey

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## ABSTRACT

The 48 hour $LD_{50}$ and symptoms of ciguatera for crude methanolic extracts of flesh and viscera from toxic surgeonfish (*Ctenochaetus strigosus*) did not differ from the toxicologic effects of identically prepared extracts from the viscera of a freshwater fish (*Tilapia sp.*), viscera from randomly collected *C. strigosus*, viscera from guinea pigs, or from the flesh of the avocado. The $LD_{50}$ of crude extract from toxic fish viscera was 468 mg/kg, while that of the other viscera and avocado was between 1400 and 1600 mg/kg. The results show that the extraction procedure yields lipid soluble toxic materials regardless of source which differ only in potency, are not necessarily involved in ciguatera, and could not be differentiated from extracts from toxic fish by the mouse bioassay.

# INTRODUCTION

Ciguatera, a syndrome occurring commonly throughout tropical regions, is characterized in man by variable but often severe symptomatology and low mortality (1). The toxin enters the food chain from a dinoflagellate, *Gambierdiscus toxicus*, and ultimately affects man after consumption of toxic fish. Since the toxin is not destroyed by cooking and cannot be detected by a practical assay in affected fish, ciguatera has become a serious public health problem in tropical oceanic regions of the world. The lack of an animal model specific for ciguatera is at least partly responsible for the difficulty in developing a specific assay for the causal toxin or toxins in fish flesh. Although the toxins are lethal to several species (2-4), the mouse bioassay utilizing a rating system based on signs, symptoms and lethal effects is the standard for detection of ciguateric toxins in the flesh and viscera of suspect fish (4,5). We examined the specificity of this assay by comparing the toxicological effects of crude methanolic extracts from fish implicated in ciguatera with extracts from species not known to cause ciguatera.

## MATERIALS AND METHODS

A crude methanolic extract (4) of flesh and viscera from the surgeonfish, *Ctenochaetus strigosus*, implicated by the Hawaii State Department of Health in an outbreak of ciguatera served as the standard for comparison with extracts prepared identically from the flesh and viscera of *Tilapia sp.*, a land-locked freshwater fish caught on the Manoa Campus of the University of Hawaii, flesh and viscera from *C. strigosus* harvested randomly in the waters of Oahu, Hawaii, viscera from Hartley guinea pigs, and flesh from avocado and beef liver obtained from a local supermarket. The extraction yield ranged from 30mg/g tissue for *Tilapia sp.* viscera to 0.7mg/g for avocado. Swiss-Webster mice of either sex weighing 25±2g were given i.p. 0.5ml of the extract solubilized in 2% Tween 60 in 0.9% NaCl. The residue of an extraction without tissue was injected into mice to control for impurities in the re-agents.

## RESULTS

The 48 hr mortality:dose relationship for four crude extracts is shown in Figure 1. The $LD_{50}$ for the extract prepared from toxic *C. strigosus* flesh and viscera was 468mg crude extract/kg. The $LD_{50}$ for the extracts prepared from *Tilapia sp.* viscera, guinea pig viscera and from avocado did not differ statistically and were between 1400 and 1600 mg crude extract/kg. Extracts prepared from beef liver and *Tilapia sp.* flesh were not lethal in the doses used. The cause of death for all lethal extracts appeared to be respiratory arrest. In some experiments, mice were observed for up to 72 hours after injection with crude extract. All mice which received $LD_{50}$ doses or greater of lethal extracts died within this period.

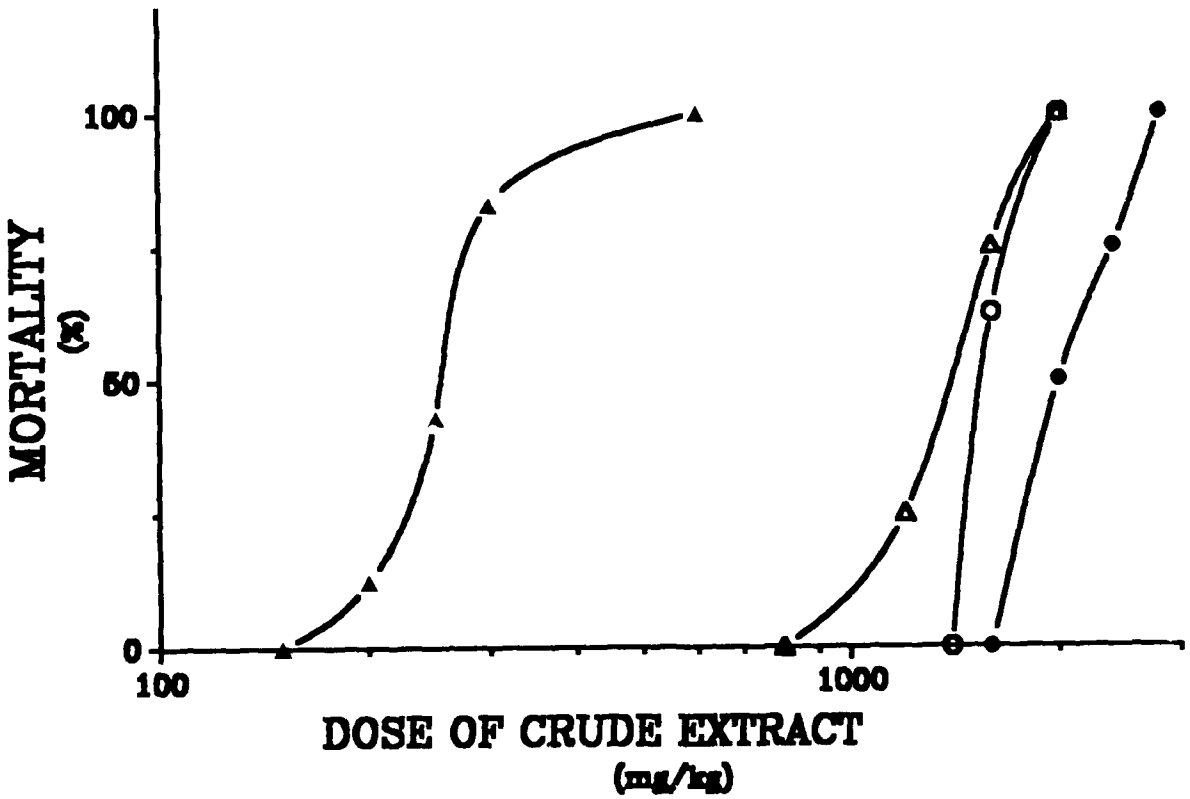


Figure 1. Forty eight hour mortality-dose relationships for crude extracts from four tissue sources. Mortality of mice to crude extract prepared from *C. strigosus viscera linked to an occurrence of ciguatera is shown by* (▲). *Mortality to extracts from nontoxic species are shown by* (△) *for Tilapia sp.* viscera, (○) for guinea pig viscera, and (●) for avocado. Each point on the figure represents at least four mice.

Toxic responses to several extracts from non-toxic species was evaluated at or above the $LD_{50}$ values determined above. The subjective symptoms of toxicity described by Kimura and coworkers (4) and Hoffman and colleagues (5) occurred to some degree in all mice regardless of the source of the extract (Table 1).

**Table 1. Bioassay Ratings for Crude Extracts Obtained from Nontoxic Sources**

| Source | Dose[a] | Mortality | Bioassay Rating[b] |
|---|---|---|---|
| *C. strigosus*[c] | | | |
| Flesh | 1645 ± 135 | 1/3 | 4,3,3 |
| Viscera | 1568 ± 127 | 4/4 | 5,5,5,5 |
| *Tilapia sp.* | | | |
| Flesh | 1610 ± 28 | 0/2 | 1,1 |
| Viscera | 1468 ± 82 | 6/6 | 5,5,5,5,5,5 |
| Guinea Pig | | | |
| Viscera | 1605 ± 87 | 3/7 | 5,5,4,3,3,3,2 |
| Avocado | 2220 ± 46 | 5/8 | 4,4,4,4,4,3,3,2 |
| Beef Liver | 2150 | 0/3 | 1,1,0 |
| Vehicle | - | 0/8 | 0,0,0,0,0,0,0,0 |

a Mean ± SEM for 1 to 5 extracts (mg crude extract/kg mouse)
b Symptomatology rated from 0 = no symptoms during eight hours to 5 = Death within six hours (4).
c Obtained by random collection in the waters of Oahu, Hawaii.

All animals except those injected with vehicle or reagent extract exhibited some degree of lethargy, ataxia and immobility regardless of the extract and survival. Some mice exhibited hyperexcitabilty when disturbed. Extracts of the flesh of *Tilapia sp.* and beef liver produced less severe syptomatology than extracts from all other species. Frequent defecation or diarrhea occurred with all tissue extracts. None of the subjective signs was specific for the extract prepared from *C. strigosus* believed to have caused ciguatera in humans. The flesh of non-toxic *C. strigosus* and *Tilapia sp.* produced milder symptomatology than the viscera of each fish.

## DISCUSSION

The results of this study show that crude tissue extracts prepared from several species not involved in ciguatera affected mice in a manner not different from that of extracts from toxic fish (4) or purified toxins (5). In contrast, Hoffman and coworkers (5) found no toxic symptomatology in purified extracts from nontoxic fish when compared to extracts from toxic fish of the same species. The difference in the dose range investigated may offer an explanation for this apparent discrepancy. The maximum dose used by Hoffman and colleagues was approximately 30% greater than the $LD_{50}$ because of the narrow dose range for lethal effects and toxicity ratings (5). We also observed a narrow lethal dose range, but found that the $LD_{50}$ for extracts from nontoxic species was approximately three times greater than that from toxic *C. strigosus*. Thus, we investigated a dose range of crude extracts which covered both $LD_{50}$s and found similar symptomatology. We conclude from these results that the crude methanolic extract, regardless of source, contained lipid soluble toxic materials which differ in potency and are not necessarily involved in ciguatera. These findings suggest, furthermore, that the specificity of the mouse bioassay for ciguatera toxins is uncertain.

## ACKNOWLEDGMENTS

Dr. Yoshitsugi Hokama generously supplied the extract from toxic *C. strigosus.*

## SELECTED REFERENCES


1. Bagnis, R., Kuberski, T. and Laugier, S. (1979) Clinical observations on 3,009 cases of ciguatera (fish poisoning) in the South Pacific. Amer.J.Trop.Med.Hyg. 28,1067,

2. Banner, A.H., Sasaki, S., Helfrich, P. and Alender, C.B. (1961) Bioassay of ciguatera toxin. Nature 189,229,

3. Lewis, R.J. and Endean, R. (1983) Occurrence of a ciguatoxin-like substance in the Spanish Mackerel (Scomberomorus commersoni). Toxicon 21,19,

4. Kimura, L.H., Hokama, Y., Abad, M.A., Oyama, M. and Miyahara, J.T. (1982) Comparison of three different assays for the assessment of ciguaoxin in fish tissues:radioimmunoassay, mouse bioassay, and in vitro guinea pig atrium assay. Toxicon 20,907,

5. Hoffman, P.A., Granade, H.R. and McMillan, J.P. (1983) The mouse ciguatoxin bioassay: A dose-response curve and symptomatology analysis. Toxicon 21,363,

Annual and Final Report

Yoshitsugi Hokama

Department of Pathology
University of Hawaii

Abstract

From August 1986 through June 1987 we have examined several hundred fishes obtained from DAR, DOH and private sources, by the stick enzyme immunoassay. The S-EIA has demonstrated that it can distinguish contaminated toxic fishes from negative or non-toxic fishes. No false negatives have occurred since the inception of the tests in 1978, by use of the immunological approach with specific antibodies to ciguatoxin and related polyethers. The S-EIA test is in the collaborative study stage under the auspices of the AOAC and IUPAC.

We have examined the G. toxicus and C. strigosus (herbivore) extracts, the first step in the ecological food chain, by biological tests and found them to be similar, but with some differences. The latter will require further assessment and structural analysis.

Technical Report (Hokama)

## Statement of the Problem

The principal goals of this part of the multidisciplinary program have been to evaluate the stick enzyme immunoassay (S-EIA) procedure for ciguatoxin and its related polyether toxins directly from fish tissues and to assess changes of crude extracts of Gambierdiscus toxicus in liver homogenates of herbivores such as Ctenochaetus strigosus. The metabolic changes carried out in vitro studies and the results were assessed both by pharmacological and S-EIA procedures.

## Background

The assessment of ciguatoxin and related polyether toxins directly from fish tissues became a reality using an immunological approach in 1977 (Hokama et al., 1977). Since then the enzyme immunoassay has been developed (see review, Hokama and Miyahara, 1986). A simplified stick enzyme immunoassay (Hokama, 1985; Hokama et al., 1987) has been devised and presently is being evaluated in a collaborative study by several laboratories (Australia, Japan, Fiji and the U.S.A.).

## Approach to Sources of Samples

Fishes for analysis by the S-EIA test have been from the following sources:

(a) Department of Land and Natural Resources (DLNR): Division of Aquatic Resources: These samples include several species and were obtained from the Island of Oahu (Table 1).

(b) Department of Health (DOH): All fishes implicated in ciguatera poisoning (based on clinical symptoms). This data is compiled in Table 2.

(c) Miscellaneous Sources: Fishes from private sports fishermen (Table 3).

2. Test Procedure

The modified stick enzyme immunoassay (Hokama, 1985) system was used (annual report, this grant, August 1986, p. 19).

3. Metabolic Studies

Metabolic studies were assessed in vivo by comparing cultured G. toxicus extracts and C. strigosus flesh extracts in hemolysis of human RBCs, mouse toxicity and stick test values. See Miyahara's report on the results with guinea pig atrial and taenia cecum assays.

## Results and Discussion

Table 1 summarizes the results of several species of fishes obtained in 1986-87 from the DLNR and examined by the S-EIA procedure. Fishes examined were from Ewa, Barbers Point, Brown Camp and Mokumanu. The first three towns are on the Leeward side of the Island of Oahu, while Mokumanu is on the Windward side. As consistently shown by our studies the Leeward fishes tend to have a higher incidence of toxicity. Also the herbivores tend to show higher incidence of toxicity than the carnivores, except for Ewa (L. kasmira 54% rejection vs 44% C. strigosus).

The DOH samples of implicated fishes consisting of various species and the results of examination by S-EIA are shown in Table 2. Toxic fishes were obtained from several different islands (Oahu, Hawaii and Kauai) and consisted of both herbivores, omnivores and carnivores. Of the thirteen of 23 samples implicated in clinical toxicity examined, 92% were considered as toxic by the stick EIA, while the corresponding catches also showed a higher incidence than the consumed samples (Hokama et al., 1987). Eighty-five individuals were exposed to the toxic fishes and 64 showed clinical symptoms of ciguatera

poisoning. Most of the ill individuals showed gastrointestinal and neurological symptoms.

The study of miscellaneous fishes is shown in Table 3. The fishes, mostly carnivores (S. dumerili and Caranx spp.) in the negative category were consumed. No incidence of ciguatera poisoning has occurred from fishes eaten in the negative category.

The results of the biological examination of G. toxicus and C. strigosus flesh extracts showed that the following activities were similar:

(a) both extracts hemolyzed human erythrocytes at 0.05% suspension in physiological saline;

(b) both extracts were injected IP at usually 50-100 mg into Balb/C mice weighing 20 gm. (killed mice in 1-48 hrs.);

(c) both extracts reacted with the S-EIA test suggesting the polyether nature of the toxins. Table 4 presents the comparisons by the erythrocyte hemolysis assay, stick enzyme immunoassay and mouse toxicity of extracts from G. toxicus and C. strigosus: Conclusion noted in (a), (b) and (c)

## Conclusions

The stick enzyme immunoassay has been thoroughly evaluated with the examination of ciguatera poisoning implicated fishes and various other fishes. Presently, the test is being examined by eight other laboratories to assess reproducibility and repeatability under the auspices of AOAC and IUPAC.

The examination of G. toxicus and C. strigosus flesh extracts by biological assays suggests closely related toxins having polyether residues.

Publications

a) Hokama, Y. and Miyahara, J.T. 1986. Ciguatera poisoning: clinical and immunological aspects. J. Toxicol.-Toxin Reviews 5(1):25-53.

b) Hokama, Y., Shirai, L.K. and Miyahara, J.T. 1986. Seafood and ciguatera poisoning: laboratory identification methods. Lab. Management 24(4):29-40.

c) Hokama, Y., Shirai L.K., Iwamoto, L.M., Kobayashi, M.N., Goto C.S. and Nakagawa, L.K. 1987. Assessment of a rapid enzyme immunoassay stick test for the detection of ciguatoxin and related polyether toxins in fish tissues. Biol. Bull. 172:144-153.

d) Hokama, Y., Honda S., Kobayashi M., Nakagawa L., Kurihara J. and Miyahara J. 1987. Monoclonal antibodies in the detection of ciguatoxin (CTX) and related polyethers in contaminated fish tissue. Asia-Pacific Congress on Animal Plant and Microbial Toxins, Singapore, 1987. In press.

e) Hokama, Y., Nakagawa L., Kobayashi, M., York, R., Kurihara, J. and Miyahara, J. 1987. The similarity between cultured Gambierdiscus toxicus (T39) and flesh extract of Ctenochaetus strigosus (herbivore). Asia-Pacific Congress on Animal Plant and Microbial Toxins, Singapore, 1987. In press.

f) Miyahara, J.T., Kamibayashi, C.K., Shirai, L.K. and Hokama, Y. 1987. The similarity of toxins in Ctenochaetus strigosus and cultured Gambierdiscus toxicus. Asia-Pacific Congress on Animal Plant and Microbial Toxins, Singapore, 1987. In press.

g) Campbell, B., Nakagawa, L.K., Kobayashi, M.N. and Hokama, Y. 1987. Gambierdiscus toxicus in gut content of the surgeonfish Ctenochaetus strigosus (herbivore) and its relationship to toxicity in tissue. Toxicon. In press.

Literature Cited

1. Hokama, Y., Banner, A.H. and Boylan, D.A. 1977. A radioimmunoassay for the detection of ciguatoxin. Toxicon 15:317.

2. Hokama, Y., Abad, M.A. and Kimura, L.H. 1983. A rapid enzyme immunoassay (EIA) for the detection of ciguatoxin in contaminated fish tissues. Toxicon 21:817.

3. Hokama, Y. 1985. A rapid simplified enzyme immunoassay stick test for the detection of ciguatoxin and related polyethers from fish tissues. Toxicon 23:939.

4. Hokama, Y., Shirai L.K., Iwamoto, L.M., Kobayashi, M.N., Goto, C.S. and Nakagawa, L.K. 1987. Assessment of a rapid enzyme immunoassay stick test for the detection of ciguatoxin and related polyether toxins if fish tissues. Biol. Bull. 172:144.

5. Bailey, L.E., Hokama, Y., Miyahara, J.T. and Scheuer, P.J. 1986. A Multi-disciplinary study of ciguatoxin and related low molecular weight toxins from marine sources. Annual Report, August 1986. U.S. Army Medical Research and Development Command. DAMD17-85-C-5236.

6. Hokama, Y. and Miyahara, J.T. 1986. Ciguatera poisoning: clinical and immunological aspects. J. Toxicol.-Toxin Reviews 5(1):25.

Table 1. Barbers Point Study - DLNR

| | TOT # | #+/± | #- | % Reject |
|---|---|---|---|---|
| Ewa | | | | |
| C. strigosus | 130 | 57 | 73 | 44 |
| L. kasmira | 84 | 45 | 39 | 54 |
| M. kuntee | 6 | 2 | 4 | 33 |
| A. dussumieri | 3 | 2 | 1 | 66 |
| P. porphyreus | 3 | 1 | 2 | 33 |
| P. multifasciatus | 4 | 3 | 1 | 75 |
| TOTAL | 230 | 110 | 120 | 48 |
| Barbers Point | | | | |
| C. strigosus | 91 | 63 | 28 | 69 |
| L. kasmira | 41 | 18 | 23 | 44 |
| M. murdjan | 20 | 14 | 6 | 70 |
| A. olivaceus | 3 | 3 | 0 | 100 |
| A. nigroris | 3 | 3 | 0 | 100 |
| M. kuntee | 2 | 0 | 2 | 0 |
| T. ballieui | 1 | 1 | 0 | * |
| P. multifasciatus | 1 | 1 | 0 | * |
| B. bilunulatus | 1 | 1 | 0 | * |
| TOTAL | 163 | 59 | 104 | 64 |
| Brown Camp | | | | |
| C. strigosus | 79 | 47 | 32 | 60 |
| A. nigroris | 11 | 3 | 8 | 27 |
| P. bifasciatus | 2 | 1 | 1 | 50 |
| C. verator | 1 | 0 | 1 | * |
| P. multifasciatus | 1 | 0 | 1 | * |
| L. kasmira | 1 | 1 | 0 | * |
| TOTAL | 95 | 43 | 52 | 55 |
| Mokumanu | | | | |
| C. strigosus | 20 | 9 | 11 | 45 |
| M. kuntee | 3 | 0 | 3 | 0 |
| A. nigroris | 2 | 0 | 2 | 0 |
| A. dussumieri | 1 | 1 | 0 | * |
| TOTAL | 26 | 16 | 10 | 38 |
| GRAND TOTAL | 514 | 238 | 276 | 54 |

Table 2. DOH Clinical Cases

| | SOURCE | #Ill/#Exp | #+/± | #- |
|---|---|---|---|---|
| A. dussumieri | Oahu | 1/1 | 0 | 1 |
| Corresp. | | | | |
| A. dussumieri | | | 0 | 1 |
| Caranx sp. | | | 0 | 1 |
| A. dussumieri | Oahu | 2/2 | No sample | |
| Corresp. | | | | |
| A. dussumieri | | | 2 | 0 |
| C. hippuris | Restaurant | 1/? | 1 | 0 |
| Caranx sp. | Hawaii | 2/5 | 1 | 0 |
| C. argus | Hawaii | 1/1 | No sample | |
| Corresp. | | | | |
| C. argus | | | 1 | 0 |
| C. strigosus | Kauai | 1/1 | No sample | |
| Corresp. | | | | |
| C. strigosus | | | 29 | 20 |
| Caranx sp. | Kauai | 4/6 | 1 | 0 |
| A. furcatus | Oahu | 2/2 | 1 | 0 |
| D. macrosoma | Market | 3/3 | No sample | |
| Corresp. | | | | |
| D. macrosoma | | | 11 | 0 |
| Fam. Serranidae | Oahu | 1/1 | 1 | 0 |
| B. bilunulatus | Hawaii | 1/5 | 1 | 0 |
| Fam. Labridae | Hawaii | | 1 | 0 |
| Corresp. | | | | |
| Caranx sp. | | | 0 | 1 |
| C. pinnulatis | | | 0 | 1 |
| Parupenaeus sp. | Maui | 2/2 | 1 | 0 |
| Caranx sp./N. chaptalli | Hawaii | 15/20 | No sample | |
| Corresp. | | | | |
| Parupenaeus sp. | | | 1 | 0 |
| R. brighami | | | 1 | 1 |
| C. rhodochrous | Hawaii | 3/3 | No sample | |
| Corresp. | | | | |
| C. rhodochrous | | | 1 | 0 |
| L. kasmira | | | 1 | 0 |
| Parupenaeus sp. | | | 0 | 1 |

Table 2 contd.

| | SOURCE | #Ill/#Exp | #+/± | #- |
|---|---|---|---|---|
| C. strigosus | Maui | 1/2 | No sample | |
| Corresp. | | | | |
| C. strigosus | | | 1 | 0 |
| A. sandvicensis | Oahu | 5/5 | 3 | 0 |
| C. strigosus | Oahu | 2/3 | No sample | |
| Corresp. | | | | |
| A sandvicensis | | | 6 | 1 |
| Thalassoma sp. | | | 1 | 0 |
| Priacanthus sp. | | | 1 | 0 |
| Parupenaeus sp. | | | 0 | 1 |
| C. strigosus | Oahu | 2/2 | No sample | |
| Corresp. | | | | |
| C. strigosus | | | 4 | 1 |
| A. sandvicensis | | | 3 | 0 |
| Parupenaeus sp. | | | 1 | 2 |
| N. brevirostris | | | 1 | 0 |
| A. dussumieri | | | 1 | 0 |
| C. strigosus | Oahu | 2/2 | No sample | |
| Corresp. | | | | |
| C. strigosus | | | 0 | 6 |
| Parupenaeus sp. | | | 3 | 1 |
| S. dumerili | | | 0 | 2 |
| L. kasmira | | | 1 | 0 |
| Mulloidichthys sp. | Oahu | 4/5 | No sample | |
| Corresp. | | | | |
| Mulloidichthys sp. | | | 0 | 2 |
| P. microlepis | Market | 2/3 | 1 | 0 |
| Mugil cephalus | Kauai | 3/4 | No sample | |
| Corresp. | | | | |
| Mugil cephalus | | | 1 | 2 |
| C. strigosus | Kauai | 4/7 | No sample | |
| Corresp. | | | | |
| C. strigosus | | | 1 | 0 |

Total # of Persons Ill/Exposed = 64/85
Total # of Cases In vestigated = 23

| | Tot # | #- | #+ | %Reject |
|---|---|---|---|---|
| Consumed Samples | 13 | 1 | 12 | 92 |
| Corresp. Samples | 119 | 44 | 75 | 63 |
| TOTAL | 132 | 45 | 87 | 66 |

Table 3. Miscellaneous Fishes

| | TOT # | #+/± | #- | % Reject |
|---|---|---|---|---|
| S. dumerili | 61 | 41 | 20 | 67 |
| Caranx sp. | 55 | 33 | 22 | 60 |
| E. bipinnulatis | 97 | 29 | 68 | 30 |
| S. barracuda | 7 | 2 | 5 | 28 |
| Mulloidichthys spp. | 84 | 62 | 22 | 74 |
| L. bohar | 10 | 7 | 3 | 70 |
| N. chaptalli | 7 | 0 | 7 | 0 |
| C. strigosus | 55 | 49 | 6 | 89 |
| A. vulpes | 5 | 2 | 3 | 40 |
| Acanthurus spp. | 20 | 8 | 12 | 40 |
| Chaetodon spp. | 3 | 3 | 0 | 100 |
| B. bilunulatis | 2 | 1 | 1 | 50 |
| Thalassoma spp. | 10 | 9 | 1 | 90 |
| U. arge | 158 | 75 | 83 | 47 |
| C. rhodochrous | 4 | 3 | 1 | 75 |
| Parupenaeus spp. | 2 | 2 | 0 | 100 |
| K. cinerescens | 1 | 1 | 0 | * |
| Myripristis spp. | 4 | 3 | 1 | 75 |
| K. sandvicensis | 8 | 3 | 5 | 37 |
| Conger sp. | 1 | 0 | 1 | * |
| P. spilosoma | 27 | 7 | 20 | 26 |
| TOTAL | 621 | 281 | 340 | 55 |

Table 4. G. toxicus and C. strigosus Extract Comparison

| FRACTION | RBC HEMOLYSIS | | S-EIA VALUE | MOUSE BIOASSAY |
|---|---|---|---|---|
| | 1000 ug | 100 ug | 10 mg/ml | Dose/Mouse (hrs) |
| G. toxicus | | | | |
| Original | - | 100% | 2.0±0.2 | 0500 ug ( 02.0) |
| Chloroform | 100% | 100% | 0.4±0.2 | 1000 ug ( 02.5) |
| | | | | 0500 ug ( 04.0) |
| Methanol | 100% | 034% | 1.3±0.6 | 1000 ug ( 07.0) |
| | | | | 0500 ug ( 24.0) |
| Aqueous | 000% | 000% | 1.4±0.3 | 1000 ug (survived) |
| | | | | 0500 ug (survived) |
| C. strigosus | | | | |
| Flesh | 100% | 100% | 1.7±0.3 | 0050 ug ( 16.0) |
| Visceral | 100% | 100% | 2.3±0.5 | 0050 ug ( 03.0) |

Acid-Base Inactivation

| | Treatment | % Inactivation |
|---|---|---|
| G. toxicus | | |
| Methanol | Acid-HAc | 065 |
| | Base | 100 |
| C. strigosus | | |
| Flesh | Acid-HAc | 100 |
| | Base | 100 |

Annual and Final Report

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ABSTRACT

Biological activities of toxic extracts of two ciguateric fishes, Ctenochaetus strigosus and Seriola dumerili, were studied on human erythrocyte hemolysis, and on the isolated guinea-pig atria and taenia caecum and compared to those of cultured Gambierdiscus toxicus and "pure" ciguatoxin (CTX).

With the exception of CTX, maitotoxin (MTX) and all the toxic extracts showed RBC hemolytic activity.

Both fish extracts also elicited similar positive inotropic and chronotropic effects on the isolated atria; however, the cardiotonic effects differed in their onset, time to peak and duration and in their response to pharmacological intervention. The inotropic effect of C. strigosus extract was slow to rise but was of long duration similar to that of G. toxicus, although the response of the dinoflagellate extract was not antagonized by tetrodotoxin (TTX). In contrast, S. dumerili extract was more CTX-like with a rapid time course and the inotropicity blocked by TTX, but little affected by adrenoceptor blockade.

Both C. strigosus and G. toxicus extracts effected a long lasting relaxation of the taenia caecum and depression of perivascular nerve-mediated (PVS) inhibitory response, at low frequencies for C. strigosus and at all frequencies for G. toxicus. On the other hand, the extract of S. dumerili only caused contraction of the muscle strip, but had no effect on the PVS-induced relaxation of the taenia caecum. These data demonstrate the presence of different toxins in different species of ciguateric fishes.

Some of the results of this study were presented at the 1st Asia-Pacific Congress on Animal, Plant and Microbial Toxins in Singapore, June 1987.

## BACKGROUND AND STATEMENT OF THE PROBLEM

Ciguatoxin (CTX), a lipid soluble toxin elaborated by the dinoflagellate Gambierdiscus toxicus, is believed the principal agent responsible for ciguatera (Scheuer et al., 1967). According to the food chain theory of ciguatera, CTX is first acquired by reef herbivores and is passed on to the carnivores and omnivores as they feed on the ciguateric fishes (Randall, 1958). However, evidence accumulated over the years would suggest several toxins may cause or contribute to this common fish poisoning. For example, even though the general symptoms of ciguatera are categorically similar, they can vary widely depending on the species of fish involved and on the site of capture. Ciguatera caused by herbivores has reportedly been associated with more severe gastrointestinal complaints, whereas the neurologic and cardiovascular effects often predominate in the poisonings by piscivores (Bagnis, 1968). While CTX is the main toxin extracted from the moray eel (Yasumoto and Scheuer, 1969), different toxins such as MTX and scaritoxin have also been found in other ciguateric fishes (Yasumoto et al., 1980; Bagnis et al., 1981; Withers, 1982).

In Hawaii among the fishes most frequently implicated in ciguatera are the surgeon fish C. strigosus and the amberjack S. dumerili. This has provided us an opportunity to examine the toxin content of two distinct ciguateric fishes and test whether a herbivore and a carnivore accumulate the same toxin or toxins. Therefore, the present investigation was undertaken to study the properties of these extracts from C. strigosus and S. dumerili and to compare them to those of cultured G. toxicus and of Scheuer's classical CTX. This report is mainly concerned with their effects on human erythrocytes and on the isolated guinea-pig atria and taenia caecum.

## MATERIALS AND METHODS

*C. strigosus* and *S. dumerili*, taken from regions of ciguatera outbreaks, were obtained from the Department of Health of the State of Hawaii. Crude extracts were prepared from the flesh of fish determined to be toxic by the stick test (Hokama, 1985) and only those that killed mice within 24 hr were used in the study. The extraction scheme was essentially that of Kimura *et al.* (1982).

Minced tissue (1.5 kg)
↓ Acetone at room temp
↓ 2x filtration

Acetone filtrate
↓ Evaporate 37° C

Aqueous mixture
↓ Extract with $CHCl_3$
↓ Backwash with 20% brine
↓ Discard brine
↓ Concentrate $CHCl_3$

$CHCl_3$ soluble fraction
↓ Extract with hexane
↓ Backwash with aqueous MeOH (80%)
↓ Discard hexane
↓ Concentrate MeOH

Crude extract (5.2 g)

The *G. toxicus* extract was prepared from the aqueous fraction of an axenic culture, T39, obtained from the Hawaii Institute of Marine Biology.

Hemolysis of human erythrocytes was determined by addition of varying concentrations of toxic extracts to a 0.05% suspension of RBCs in phosphate buffered saline. Whole venous blood was collected in a heparinized tube, the RBCs packed by centrifugation and a stock suspension of 0.5% RBC was made in 0.85% saline. Aliquots (0.9 ml) of this RBC suspension were placed in incubation tubes, 0.1 ml of the toxic extract was added to each tube and the tubes were incubated at room temperature for 18 to 24 hr. From each tube 0.3 ml of the

supernatant was taken and placed in microtiter wells and read on a spectrophotometer at 550 nm. The results were expressed as % hemolysis based on the controls, no hemolysis (1.0 ml 0.05% RBC only) and 100% hemolysis (0.9 ml 0.05% RBC + 0.1 ml $H_2O$).

Adult male guinea-pigs were used in these experiments; the heart was removed and the right and left atria were dissected and mounted in 25 ml tissue baths with Krebs-bicarbonate solution (bubbled with 95% $O_2$ - 5% $CO_2$, pH 7.4 at 30$^{o}$ C). The right atrium was suspended in a chamber to record the spontaneous contractions; the hemi strips of the left atrium were prepared and secured onto a plexiglass tissue holder imbedded with platinum electrodes designed for electrical stimulation of small tissues. Atrial contraction and developed tension were recorded isometrically and displayed on a Grass polygraph.

Effects of the toxin extracts on the taenia caecum were studied on the adrenergically-mediated inhibitory system. The nerve-smooth muscle preparation was obtained by dissecting a segment of the taenia caecum together with the perivascular nerves which traverse along the mesentary artery supplying the muscle. Stimulation of the adrenergic pathway was secured by supramaximal rectangular pulses (1.5 msec) delivered to the perivascular nerve for 20 sec at varied frequencies and was revealed by the relaxation of tne taenia caecum.

## RESULTS AND DISCUSSION

The major toxins of ciguatera are CTX and MTX: the first is lipoidal and extracted from tissues of ciguateric fish; the second is water soluble and obtainable from cultured G. toxicus, the presumed progenitor of CTX in nature. In a separate study Hokama et al. (1987) reconfirmed that MTX, but not CTX, concentration dependently (10 to 100 ng/ml) hemolyzed human erythrocytes. Based on this finding it was thought this difference in hemolytic activity might serve as a useful test to distinguish the toxins in our toxic fish

extracts. The effects of the fish extracts along with that of G. toxicus on RBC hemolysis are shown in Table 1. In this study both extracts of C. strigosus and S. dumerili (at concentrations in excess of that required for the atrial effect) caused RBC hemolysis indistinguishable from that of G. toxicus. The results with the extract of G. toxicus and C. strigosus were not unexpected, since MTX is found in related surgeon fish and is also extractable from the aqueous fraction of cultured dinoflagellate as corroborated by the bimodal response on the atria (Figure 3c). However, as to the activity of S. dumerili, at this time we can only speculate the presence of MTX in this fish extract.

Table 1. Hemolytic activity of C. strigosus and S. dumerili extracts

| Toxin | Conc. (/ml) | % Hemolysis | Conc. (/ml) for atrial effect |
|---|---|---|---|
| MTX | 10 ng | 55 | 880 pg |
| | 100 ng | 100 | |
| CTX | 10 ng | 0 | 80 ng |
| | 100 ng | 0 | |
| G. toxicus (aqueous fraction) | 200 µg | 0 | 400 µg |
| | 1000 µg | 15 | |
| | 2000 µg | 100 | |
| C. strigosus | 10 µg | 67 | 400 µg |
| | 50 µg | 100 | |
| | 100 µg | 100 | |
| S. dumerili | 10 µg | 8 | 720 µg |
| | 50 µg | 75 | |
| | 100 µg | 100 | |

Both C. strigosus and S. dumerili extracts elicited CTX-like positive inotropic and chronotropic effects on the isolated atria (Figures 1a, 1b and 2a, 2b). As with CTX (Figure 4c) the effects of the fish extracts were readily counteracted by post treatment with sodium channel blockers such as tetrodotoxin (TTX) and saxitoxin (Figures 1c and 2c). However, the pattern of their responses and their pharmacology to adrenoceptor antagonists were quite different. For example, the inotropic effect of C. strigosus extract on the electrically contracted atria was slow to rise and to peak (11.8 $\pm$ 0.29 min, mean $\pm$ SEM, n = 12), but the increased contractions persisted for over an hour (Figure 1b). A similar long lasting inotropic effect was observed with a low concentration of G. toxicus extract (Figure 3b), although this response was little affected by TTX (Figure 3d). With high concentrations the G. toxicus response was bimodal with a terminal increase in resting tension (Figure 3a), characteristic of MTX as reported by Kobayashi et al. (1986). In contrast the time course of inotropicity of the S. dumerili extract was more comparable to that of classical CTX (Figure 4b), with a sharp rise, rapid time to peak (2.08 $\pm$ 0.14 min, mean $\pm$ SEM, n = 10) and relatively short duration (Figure 2b). It is possible some tissue constituent particularly in the extraction of C. strigosus, but absent in S. dumerili, effected the delay in the onset of action, but the difference in their duration of effects is better explained by the presence of different toxins in the extracts. Both toxins may have similar action on the sodium channel but with a different affinity for the receptor.

A part of the CTX inotropic effect is associated with the release of noradrenaline and is therefore depressed by adrenoceptor antagonists such as phentolamine and propranolol (Figure 4d). In this study the adrenergic blockers not only depressed the cardiotonic effect of C. strigosus extract but this antagonism occurred along the whole time course of the inotropic effect (Figure 1d). In contrast, the response of S. dumerili (Figure 2d) was altered very

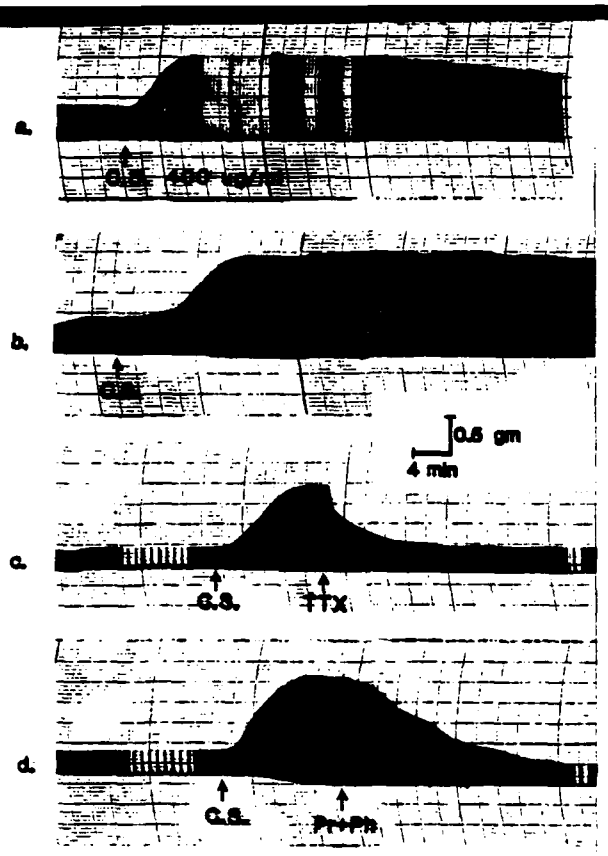


Figure 1.

Effects of toxic extract of *C. strigosus* on the isolated guinea-pig atria. (a) Rt atrium (control) (b) Lt atrium (control) (c) Lt atrium, Tetrodotoxin ($5x10^{-7}$M) (d) Lt atrium, Phentolamine ($5x10^{-7}$M) + Propranolol ($10^{-6}$M).

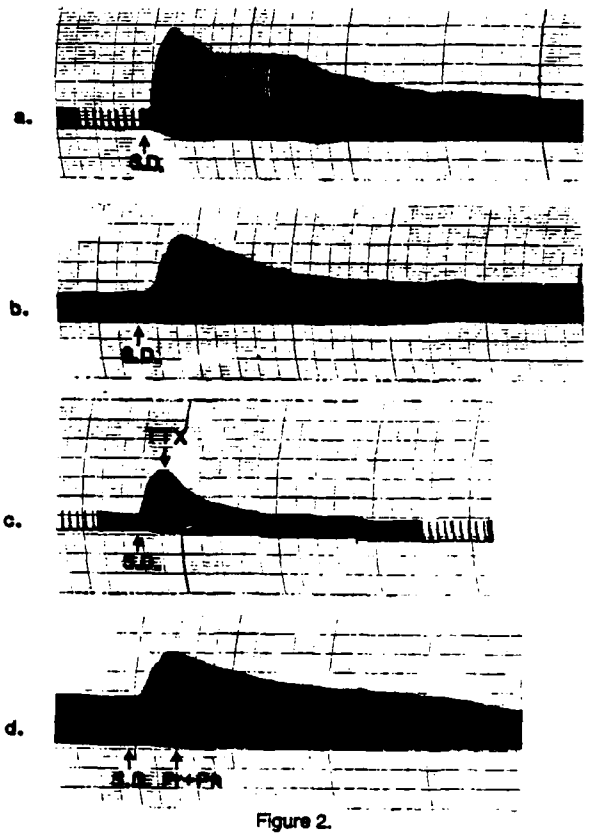


Figure 2.

Effects of toxic extract of *S. dumenil* on the isolated guinea-pig atria. (a) Rt atrium (control) (b) Lt atrium (control) (c) Lt atrium, Tetrodotoxin ($5x10^{-7}$M) (d) Lt atrium, Phentolamine ($5x10^{-7}$M) + Propranolol ($10^{-6}$M).

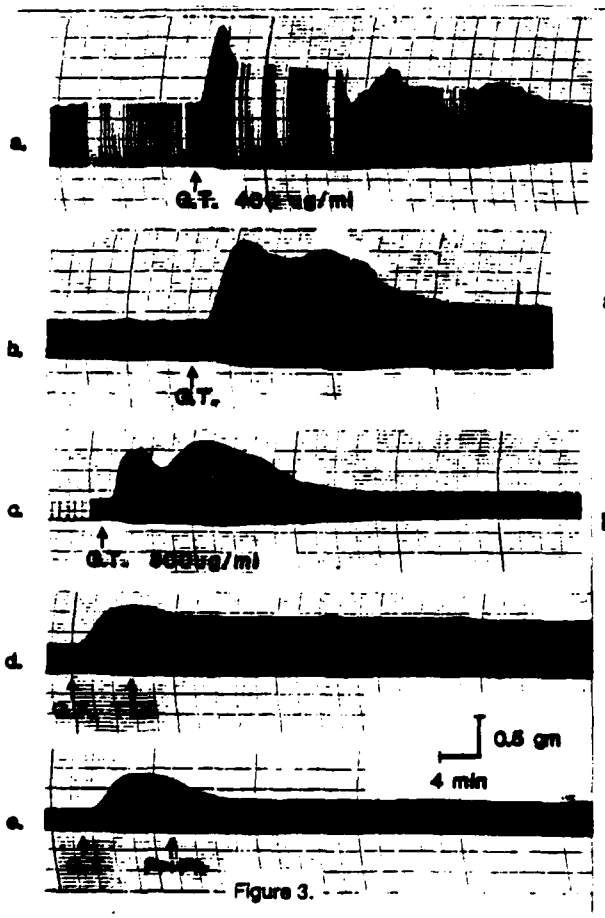


Figure 3.

Effects of cultured *G. toxicus* extract on the isolated guinea-pig atria. (a) Rt atrium (control) (b) Lt atrium, Low conc. (control) (c) Lt atrium, High conc. (control) (d) Lt atrium, Tetrodotoxin ($5x10^{-7}$M) (e) Lt atrium, Phentolamine ($5x10^{-7}$M) + Propranolol ($10^{-6}$M).

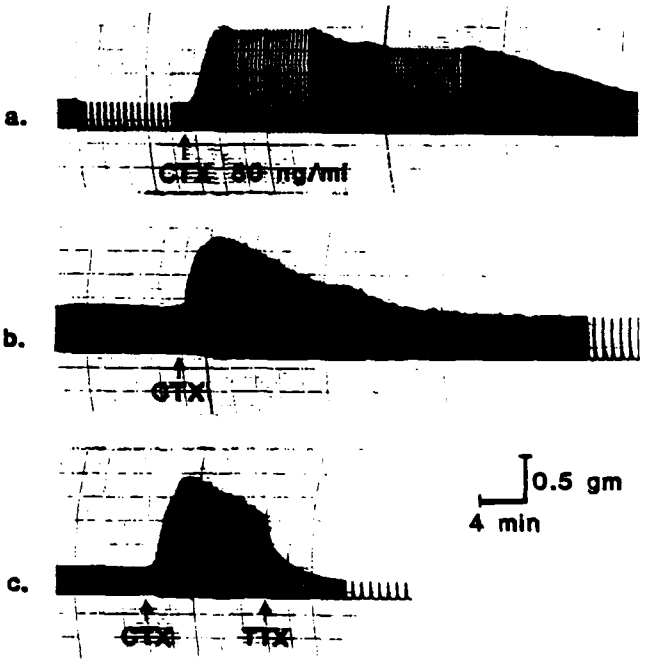


Figure 4.

Effects of CTX (*G. javanicus* ) on the isolated guinea-pig atria. (a) Rt atrium (control) (b) Lt atrium (control) (c) Lt atrium, Tetrodotoxin ($5x10^{-7}$M).

little by these agents, even when applied at the time of maximum effect. These results revealed the persistent release of catecholamines that underly the long action of C. strigosus extract and the lack of sustained transmitter function associated with the S. dumerili response.

Miyahara and Shibata (1976) reported that CTX causes an immediate release of noradrenaline, a brisk and brief relaxation of the taenia caecum and depression of the inhibitory responses evoked by low frequency perivascular nerve stimulation. In Figure 5, administration of C. strigosus extract led to a rapid relaxation of the taenia strip, a long lasting increase in spontaneous activity and like CTX, subsequent depression of PVS responses at the low but not high frequencies.

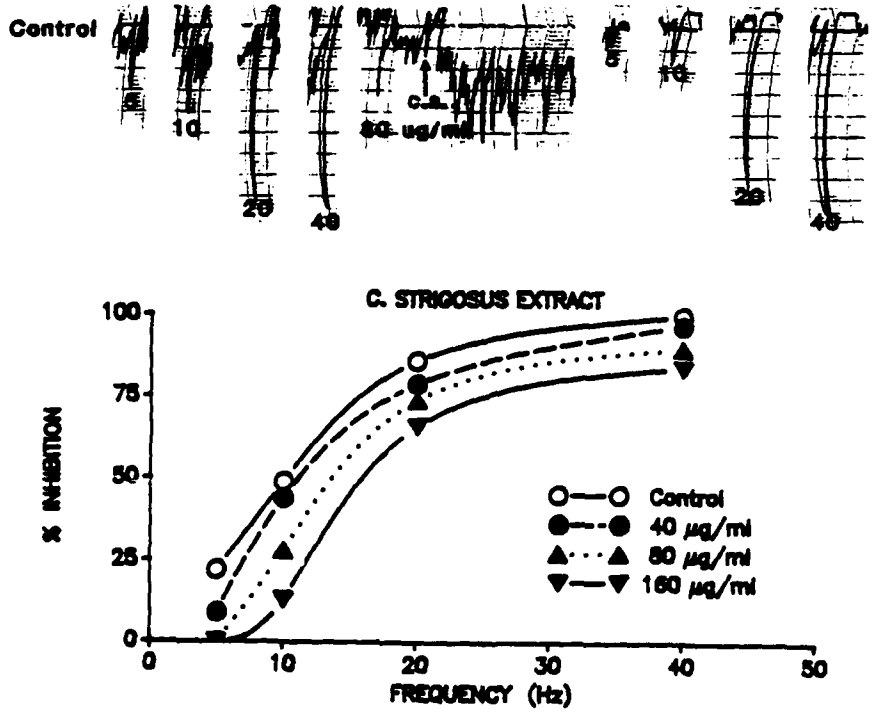


Figure 5. Frequency response curves of PVS-evoked relaxation of taenia caecum in the presence of *C. strigosus* extract.

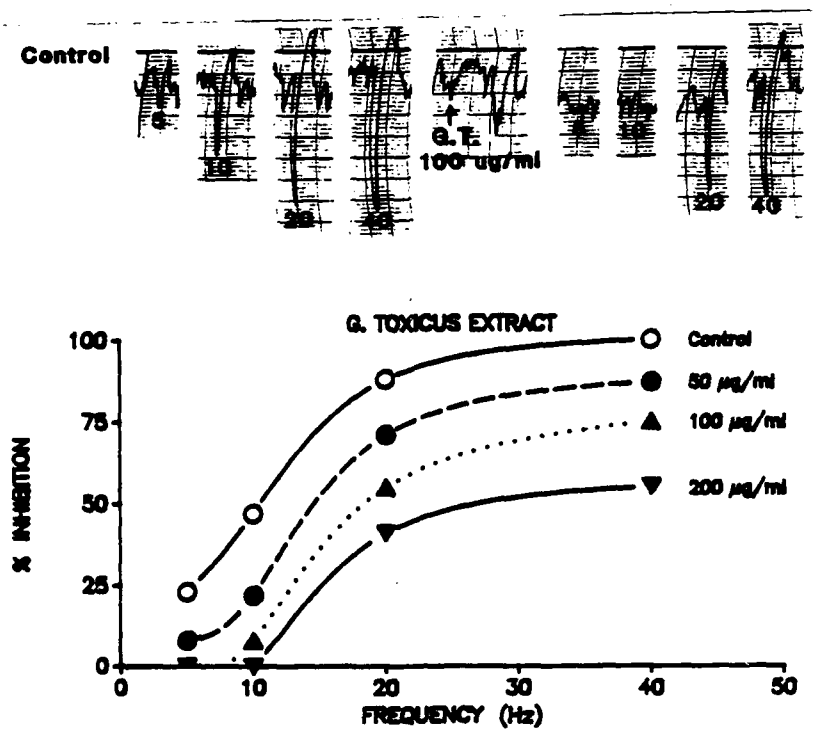


Figure 6. Frequency response curves of PVS-evoked relaxation of taenia caecum in the presence of *G. toxicus* extract.

Application of G. toxicus extract caused similar release of the inhibitory transmitter and relaxation of the taenia caecum, but the evoked response to PVS was attenuated at all stimulus frequencies (Figure 6). The effects of S. dumerili extract was quite different in that this toxic extract did not cause relaxation but contraction of the taenia strip and suppression of ongoing spontaneous activity (Figure 7). However, even at concentrations higher than that effective for the atrial response, it had little effect on the PVS-evoked relaxation. These differential effects on the intestinal smooth muscle demonstrate the different actions of the toxins in the toxic extracts of C. strigosus and S. dumerili.

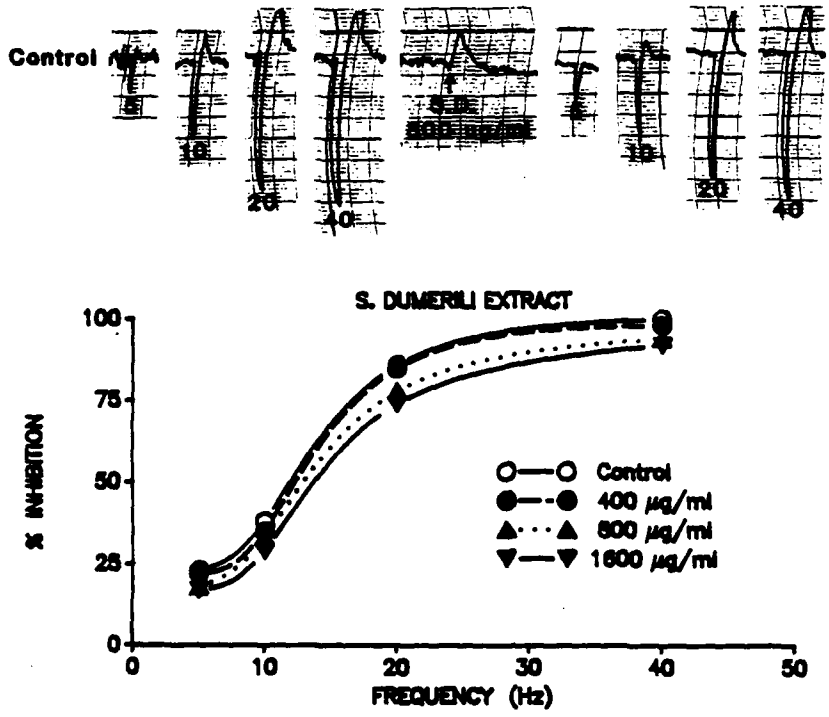


Figure 7. Frequency response curves of PVS-evoked relaxation of taenia caecum in the presence of *S. dumerili* extract.

## CONCLUSION

This study revealed both similar and dissimilar properties of the toxic extracts of C. strigosus and S. dumerili. The data in the present work suggest similar but yet different toxins are present in different species of ciguateric fishes and are supportive of the notion that the toxins of ciguatera are of multiple origin.

## LITERATURE CITED


1. Bagnis, R. (1968) Clinical aspects of ciguatera (fish poisoning) in French Polynesia. Hawaii Med. J., 28, 25.

2. Hokama, Y. (1985) A rapid, simplified enzyme immunoassay stick test for the detection of ciguatoxin and related polyethers from fish tissues. Toxicon, 23, 939.

3. Hokama, Y., Nagasawa, L., Kobayashi, M., York, R., Kurihara, J., Miyahara, J. (1987) The similarity between cultured Gambierdiscus toxicus (T39) and flesh extract of Ctenochaetus strigosus (Herbivore). First Asia Pacific Congress on Plant and Microbial Toxins, in press.

4. Kimura, L.H., Hokama, Y., Abad, M.A., Oyama, M., Miyahara, J. (1982) Comparison of three different assays for the assessment of ciguatoxin in fish tissues: Radioimmunoasssay, mouse bioassay and in vitro guinea-pig atrium assay. Toxicon, 20, 907.

5. Kobayashi, M., Kondo, S., Yasumoto, T., Ohizumi, Y. (1986) Cardiotoxic effects of maitotoxin, a principal toxin of sea food poisoning, on guinea-pig and rat cardiac muscle. J. Pharmacol. Exp. Ther., 221, 748.

6. Miyahara, J., Shibata, S. (1976) Effects of ciguatera toxin on the guinea-pig taenia caecum. Fed. Proc., 35, 842.

7. Randall, J.E. (1958) A review of ciguatera tropical fish poisoning with a tentative explanation of its cause. Bull. Mar. Sci. Gulf Caribb., 8, 236.

8. Scheuer, P.J., Takahashi, W., Tsutsumi, J., Yoshida, T. (1967) Ciguatoxin: Isolation and chemical nature. Science, 21, 1267.

9. Yasumoto, T., Scheuer, P.J. (1969) Marine toxins of the Pacific VIII. Ciguatoxin from moray eel livers. Toxicon, 7, 273.

10. Yasumoto, T., Bagnis, R., Vernoux, V.P. (1976) Toxicity of the surgeon fishes II. Properties of the principal water soluble toxin. Bull. Jpn. Soc. Sci. Fish., 42, 359.


## PUBLICATIONS DURING THIS REPORTING PERIOD


Miyahara, J.T., Kamibayashi, C.K., Shirai, L.K., Hokama, Y. (1987) The similarity of toxins in Ctenochaetus strigosus and cultured Gambierdiscus toxicus. In press.

Hokama, Y., Miyahara, J.T. (1987). Ciguatera poisoning: Clinical and immunological aspects. J. Toxic.:Toxin Rev. In press.

Annual and Final Report

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Abstract

From August 1986 through June, 1987 we received 34.9 kg of toxic eel viscera from Tarawa atoll, Republic of Kiribati. All of this material has been purified up to, but excluding the final liquid chromatography step. The expected estimated yield is 500 μg of ciguatoxin (CTX), on the basis of mouse bioassay.

We also contracted for, and received, an experimental shipment of presumed toxic fish (11.1 kg), principally *Lutjanus boher*, from Ulithi Atoll, Federated States of Micronesia. Extracts of these fish were mildly toxic, but the mice failed to exhibit ciguatera symptoms.

The most likely molecular formula of CTX is $C_{59}H_{86}NO_{19}$. If this is confirmed by a duplicate experiment, CTX is the only known polyether toxin containing nitrogen.

During the reporting period, the lectures presented at a June, 1985 Ciguatera Workshop, appeared in print (*Biol. Bull.* **1987**, *172*, 89–153). An authoritative account of ciguatera in Australia also was published (Gillespie, N.C.; Lewis, R.J.; *et al. Med. J. Aust.* **1986**, 145, 584–590).

Technical Report (Scheuer)

## Statement of the Problem

Ciguatoxin (CTX) is a major marine toxin of unknown molecular structure. It is one of the most potent molecular weight toxins ($LD_{50}$ 0.45 μg/kg, ip mice; M Wt 1,111 daltons) and is responsible for human intoxication from ingestion of coral reef fishes in many parts of the world. Since a study of its mechanism of action, the design of a specific diagnostic test, and development of a rational therapy depend entirely on availability of pure toxin and partly on a knowledge of its molecular structure, isolation, purification, and molecular structure determination are the principal goals of this part of the multidisciplinary program.

## Background

Outbreaks of ciguatera fish poisoning are unpredictable in time or place and persist for limited periods. Hence procurement of toxic fish for scientific research has been logistically difficult and unrewarding as the concentration of CTX in toxic fish ranges from 1 to 10 ppb (Tachibana, 1980). Yasumoto's discovery (Yasumoto et al., 1977) of a benthic dinoflagellate, *Gambierdiscus toxicus*, as the originating organism held initial promise that toxin procurement would be solved by laboratory culture. Yet despite vigorous efforts in Japan, France, and the United States G. *toxicus* cultures have not yielded significant amounts of the toxin (Scheuer and Bagnis, 1985). Consequently, toxic fish catches from known or suspected ciguateric areas have remained the only source of CTX.

## Approach

### (i) procurement

Because of our familiarity with the Pacific we have concentrated our procurement efforts on Pacific archipelagoes with a current ciguatera problem. Because of the distances involved and the difficulties of communication and transportation, we have attempted to set up a procurement operation by personal contact to be followed up by air shipment of toxic fish viscera. Viscera rather than flesh are known to be the best yielding source of CTX (Yasumoto and Scheuer, 1969).

### (ii) purification

In order the minimize losses during our complex multi-step purification procedure we accumulate and combine toxin prior to column and prior to high pressure liquid chromatography and we carry out bioassay monitoring only when absolutely necessary.

### (iii) molecular structure

Our molecular structure goals are being approached by two parallel avenues: high-field NMR experiments with pure toxin and crystallization attempts of a CTX p-bromobenzoate.

## Results and Discussion

### (i) procurement

Personal contacts by project personnel with knowledgeable residents in

Kiribati, French Polynesia, and New Caledonia have resulted in a resonably study supply of toxic eel viscera only from Tarawa atoll (173°E, 1°20'N), Republic of Kiribati. Since August, 1986 we have received 34.9 kg of frozen viscera with an expected estimated yield of 500 μg of CTX.

(ii) purification

All toxin has been purified up to, but excluding the liquid chromatography step.

(iii) molecular structure

The molecular formula of CTX has been determined by high resolution mass spectral experiments. It is $C_{59}H_{85}NO_{19}$ although this needs to be confirmed by a duplicate experiment. The toxin has been crystallized (Fig.1), but the crystals are too small for structural determination by x-ray diffraction techniques. A pentabromobenzoate of CTX has been prepared, but it is not crystalline.

Conclusions

The demonstration that a nitrogen atom is present in CTX is a significant achievement. A nitrogen function of as yet unknown nature sets this toxin apart from other known polyether toxins — okadaic acid, the halichondrins and the brevetoxins.

(iiii) Three reviews on some aspects of the ciguatoxin problem have been published during the past year.

a. Tachibana, K.; Nukina, M.; Joh, Y.-G.; Scheuer, P. J. 1987. Recent Developments in the Molecular Structure of Ciguatoxin. *Biol. Bull.* 172: 122-127. [Copy appended.]

b. Nukina, M.; Tachibana, K.; Scheuer, P. J. 1986. Recent Developments in Ciguatera Research. In "Natural Toxins, Animal, Plant, and Microbial" (Harris, J. B., Ed.) Clarendon Press, Oxford, pp 46-55.

c. Gillespie, N. C.,; Lewis, R. J.; Pearn, J. H.; Bourke, A. T. C.; Holmes, M. J.; Bourke, J. B.; Shields, W. J. 1986. Ciguatera in Australia *Med. J. Aust.* 145: 584-590.

Literature Cited

Scheuer, P. J. and Bagnis, R. 1985. Ciguatera and Other Reef Seafood Poisoning. Proc. Fifth Internat. Coral Reef Congress, Vol. 4 pp 401-502.

Tachibana, K. 1980. Structural Studies on Marine Toxins. Ph.D. Thesis, University of Hawaii.

Yasumoto, T. and Scheuer, P. J. 1969. Marine Toxins of the Pacific. 8. Ciguatoxin from Moray Eel Livers. *Toxicon* 7:273-276.

Yasumoto, T., Nakajima, I., Bagnis, R. and Adachi, R. 1977. Finding of a Dinoflagellate as Likely Culprit of Ciguatera. *Bull. Jap. Soc. Sci. Fish.* 43:1021-1026.

Fig. 1 Photomicrograph of crystalline CTX (Magnification approximately 30-fold; polarizing microscope; Dr. K. A. Pankiwskyj)

Reference: *Biol. Bull.* **172:** 122–127. (February, 1987)

# RECENT DEVELOPMENTS IN THE MOLECULAR STRUCTURE OF CIGUATOXIN

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## ABSTRACT

Crystalline ciguatoxin isolated from moray eel (*Lycodontis* ≡ *Gymnothorax javanicus*) viscera has an $LD_{50}$ of 0.45 g/kg (i.p., mice). It has a molecular weight of 1111.7 ± 0.2 daltons. $^1H$ NMR studies have shown that it is a polar and highly oxygenated molecule belonging to the class of polyethers. On basic alumina ciguatoxin is reversibly converted to a chromatographically distinct less polar form, which is equally toxic and elicits typical ciguatoxin symptoms in mice.

From parrotfish (*Scarus sordidus*), which originated on a ciguateric reef on Tarawa atoll (Kiribati), we have isolated two toxins that evoke ciguatera symptoms in mice at approximately equal levels. Chromatographic evidence suggests that the two toxins are identical with the two ciguatoxins of different polarity and that the less polar form is the previously described scaritoxin.

## INTRODUCTION

> "He who oversees everything also created very many poisonous fish, in this way he punishes those who seek them."
>
> —*J. Grevin*

In his book on venoms the 16th century, French physician and poet Jacques Grevin (Grevin, 1568) intuitively foresaw some of the frustration which has been the hallmark of ciguatera research during the past thirty years. Although ciguatoxin, the principal toxin in ciguateric carnivorous fish, may be superficially compared with the well-known marine toxins tetrodotoxin and the saxi-gonyautoxins, they share few characteristics with ciguatoxin. The single factor responsible for the slow progress in ciguatera research is that ciguatoxin is a slow-acting toxin which is rarely fatal to man. It is rarely fatal not because of its lack of potency—indeed its potency is surpassed only by that of palytoxin (0.15 μg/kg; Moore and Scheuer, 1971) and maitotoxin (0.2 μg/kg; Ohizumi and Yasumoto, 1983)—consumers of ciguateric fish survive because of its extremely low concentration in fish flesh. Thus, it has been difficult to accumulate enough toxin for structural research and—for many years—to convince skeptical fellow scientists of the very existence of a well-defined toxic entity. As a slowly acting toxin, ciguatoxin lacks the dramatic impact of a fast-acting toxin, somehow a convincing demonstration of the power of a toxin.

## METHODS AND RESULTS

Because of the many variables attending ciguatera outbreaks, among them place, time, species of fish, and reported symptoms in man, we have confined our research to toxin isolated from the moray eel (*Lycodontis* ≡ *Gymnothorax javanicus*), initially from flesh and viscera, but more recently exclusively from viscera in order to economize on solvents and shipping cost. Geographically, the eels originated from John-

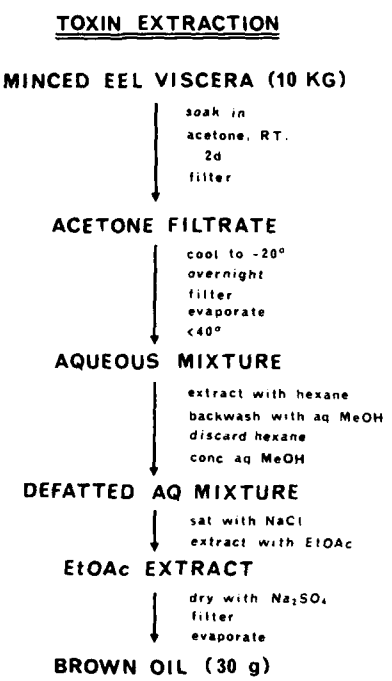



FIGURE 1. Ciguatoxin extraction.

ston atoll (165°W, 17°N) and more recently from Tarawa atoll (173°E, 1°30'N), Republic of Kiribati.

Figures 1 and 2 summarize our procedure for toxin extraction and purification (Tachibana, 1980). Approximately 75 kg of eel viscera, representing some 1100 kg of toxic eels, yielded 1.3 mg of chromatographically pure toxin. The final HPLC trace (Fig. 3) obtained on a $C_{18}$ reversed phase silica column is a symmetrical peak. The toxin, $LD_{50}$ 0.45 $\mu g/kg$ (i.p., mice), is a colorless solid readily soluble in methanol, ethanol, 2-propanol, and acetone, sparingly soluble in chloroform and diethyl ether, and nearly insoluble in water or benzene.

Ciguatoxin displays a single UV absorption peak at 215 nm ($\epsilon$ 5250). At that wavelength its CD (circular dichroism) molecular ellipticity is −620. The most prominent features in the infrared spectrum (FT, solid film) of ciguatoxin are hydroxyl (3450 $cm^{-1}$) and ether (1080 $cm^{-1}$) bands. A respectable signal at 1600 $cm^{-1}$ cannot be unequivocally interpreted.

Californium-252 plasma desorption mass spectrometry pointed to a likely molecular weight of 1111.7 ± 0.3 daltons (R. D. Macfarlane and C. McNeil, pers. comm.). Since this technique is unsuitable for high resolution measurements, no molecular formula of ciguatoxin was obtained. On the basis of $^1H$ NMR data, formulas such as $C_{53}H_{77}NO_{24}$ (1112.2) or $C_{54}H_{78}O_{24}$ (1111.2) are reasonable.

The bulk of the structural information was derived from extensive $^1H$ NMR experiments at 360 and 600 MHz in methanol-$d_4$ or dimethylsulfoxide-$d_6$. Methanol gives rise to a well-defined spectrum, but provides no clues for exchangeable protons. Ciguatoxin possesses five hydroxyl groups, four carbon-carbon double bonds, and five methyls, all on saturated carbon. The combined structural pieces account for

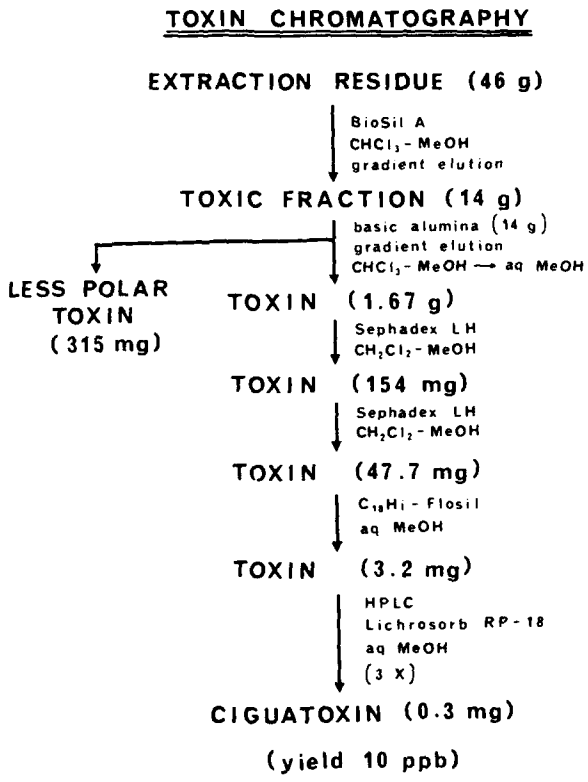



FIGURE 2. Ciguatoxin purification.

nearly 75 hydrogen atoms and imply 54 carbon atoms, but in the absence of a carbon spectrum it is impossible to judge the degree of overlap, which casts doubt on the reliability of the carbon count. Although no satisfactory carbon-13 spectrum of ciguatoxin has been determined because of lack of toxin and/or sufficient instrument time, our sample crystallized in an NMR tube during an attempt to have the carbon spectrum measured at a mainland applications laboratory. The crystals, unfortunately, are too small to be suitable for x-ray diffraction studies, and our attempts at growing larger crystals have so far failed.

## DISCUSSION AND CONCLUSION

When one considers the broad spectrum of symptoms which ciguatera-intoxicated patients describe and the difficulty of isolating a homogeneous toxin from a complex matrix in which it is present at a concentration of approximately $1 \times 10^{-6}$%, the question of multiple toxins inevitably arises. In the absence of convincing evidence to the contrary, we have followed the simplest assumption in our research; *i.e., we have assumed that ciguatoxin is a single entity.* Yet occasionally we observed (Tachibana, 1980) that in samples of extracts that had been stored for some time, ciguatoxin would be eluted from a silica column with a less polar solvent mixture (chloroform/methanol 97:3) than is the case normally, when the bulk of the toxin is eluted with a 9:1 chloroform/methanol solution. The existence of a less polar form

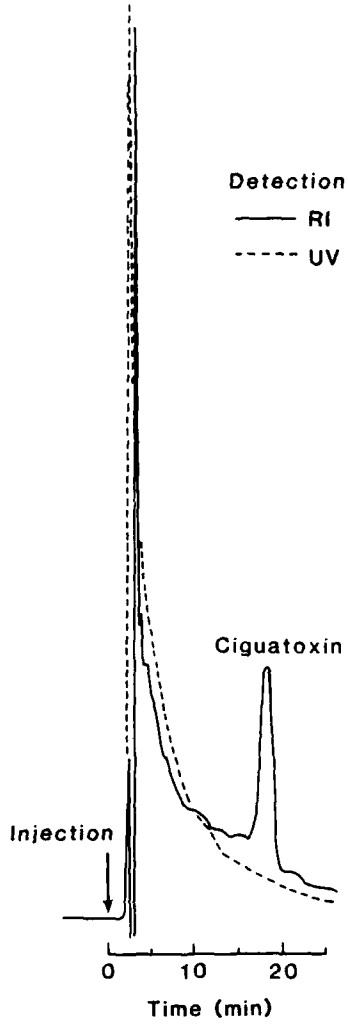
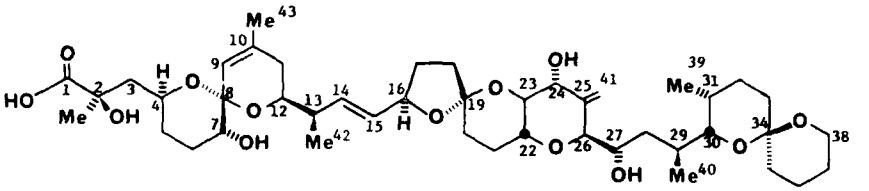



FIGURE 3. Reversed phase HPLC of ciguatoxin.

of ciguatoxin can be demonstrated by chromatography on basic alumina of different activity grades. We showed (Nukina *et al.*, 1984) that the two forms of ciguatoxin, while chromatographically distinct, are interconvertible. The two forms have $^{1}H$ NMR spectra which differ only in minor details and elicit comparable symptoms in mice.

An epidemiological survey including detailed case studies in the Gambier islands, where ciguatera intoxication arises principally from eating parrotfishes (Scaridae), led Bagnis *et al.* (1974) to suggest that either a new toxin or multiple toxins were involved. In her follow-up, Chungue (1977; Chungue *et al.*, 1977) isolated from the flesh of *Scarus gibbus* a toxic mixture which was separable by DEAE cellulose chromatography into a toxin designated scaritoxin and a more polar toxin which strongly resem-



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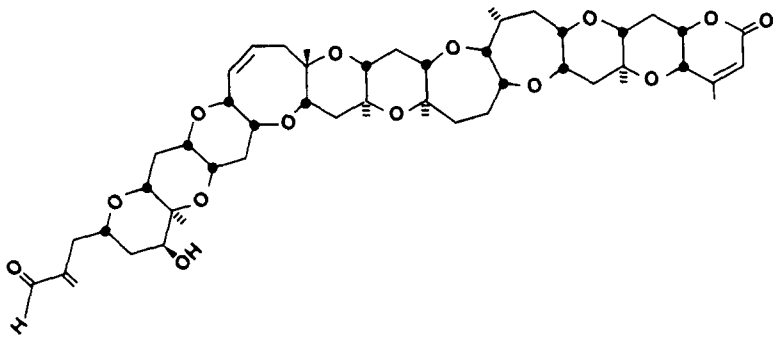

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FIGURE 4. (1) Molecular structure of okadaic acid. (2) Molecular structure of brevetoxin B.

bled ciguatoxin. Scaritoxin was reported to cause severe hind limb paralysis in mice, a symptom not normally observed with ciguatoxin.

Recently, we (Joh and Scheuer, in press) examined parrotfish (*Scarus sordidus*) from a toxic reef on Tarawa atoll, Republic of Kiribati. We also isolated two toxins separable on DEAE cellulose. However, by manipulation on basic alumina we were able to interconvert the two toxins. By TLC comparison we showed that scaritoxin and the less polar ciguatoxin (Nukina *et al.*, 1984) are identical, though this finding remains to be confirmed by spectral comparison.

When Yasumoto (Murakami *et al.*, 1982) examined the constituents of the toxic dinoflagellate *Prorocentrum lima*, he made the surprising discovery that on TLC analysis one of the constituents was indistinguishable from ciguatoxin. The substance proved not to be ciguatoxin but okadaic acid (Fig. 4, 1), a compound which we had shortly before reported from a sponge, *Halichondria okadai* (Tachibana *et al.*, 1981). The two compounds differ greatly in size (1111 *vs.* 804 daltons) and lethality (0.45 *vs.* 192 μg/kg), but evidently not in polarity because of their similar chromatographic behavior. This was the first clear indication that ciguatoxin belongs to the class of polyethers, as does, *inter alia*, brevetoxin B (Fig. 4, 2) (Lin *et al.*, 1981). These compounds are highly oxygenated long-chain fatty acids, in which most of the oxygen atoms occur as cyclic ether linkages. Okadaic acid (Fig. 4, 1), possesses one carboxyl, four hydroxyls, and seven oxa rings, in addition to three carbon-carbon double bonds. This information allows us to extrapolate safely to the ciguatoxin structure, which evidently is a complex polyether possessing five hydroxyls and four carbon-carbon double bonds. This close structural analogy to okadaic acid (Fig. 4, 1) and to brevetoxin (Fig. 4, 2) provides a plausible rationale for the interchangeability of different chromatographic forms of ciguatoxin and scaritoxin. The presence in cigua-

toxin of multiple hydroxyl groups permits formation and destruction of various hydrogen-bonded forms while preserving the structural integrity of the molecule. Because of its large size, ciguatoxin may well assume two or more secondary shapes which prevail under different conditions of basicity.

Perhaps the most intriguing questions posed upon examination of these compounds are those dealing with their mechanism of physiological action and with the subtle structural features that give rise to profound differences in lethality and overt symptoms in mammals.

## ACKNOWLEDGMENT

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## LITERATURE CITED

BAGNIS, R., E. LOUSSAN, AND S. THEVENIN. 1974. Les intoxications par poissons perroquets aux îles Gambier. *Med. Trop.* **34:** 523–527.

CHUNGUE, E. 1977. *Le Complexe Toxinique des Poissons Perroquets.* Ph.D. Thesis, Academie de Montpelier, Université des sciences et techniques du Languedoc, Montpelier, France.

CHUNGUE, E., R. BAGNIS, N. FUSETANI, AND Y. YASHIMOTO. 1977. Isolation of two toxins from a parrotfish *Scarus gibbus. Toxicon* **15:** 89–93.

GREVIN, J. 1568. *Deux Livres des Venins.* Christofle Plantin, Antwerp, Belgium.

JOH, Y.-G., AND P. J. SCHEUER. In press. The chemical nature of scaritoxin. *Marine Fish. Rev.*

LIN, Y. Y., M. RISK, S. M. RAY, J. CLARDY, D. VAN ENGEN, J. GOLIK, J. C. JAMES, AND K. NAKANISHI. 1981. Isolation and structure of brevetoxin B from the "red tide" dinoflagellate *Ptychodiscus brevis* (*Gymnodinium breve*). *J. Am. Chem. Soc.* **103:** 6773–6775.

MOORE, R. E., AND P. J. SCHEUER. 1971. Palytoxin: a new marine toxin from a coelenterate. *Science* **172:** 495–498.

MURAKAMI, Y., Y. OSHIMA, AND T. YASUMOTO. 1982. Identification of okadaic acid as a toxic component of a marine dinoflagellate *Prorocentrum lima. Nippon Suisan Gakkaishi* **48:** 69–72.

NUKINA, M., L. M. KOYANAGI, AND P. J. SCHEUER. 1984. Two interchangeable forms of ciguatoxin. *Toxicon* **22:** 169–176.

OHIZUMI, Y., AND T. YASUMOTO. 1983. Contraction and increase in tissue calcium content induced by maitotoxin, the most potent known, marine toxin, in intestinal smooth muscle. *Br. J. Pharmacol.* **79:** 003–005.

TACHIBANA, K. 1980. *Structural Studies on Marine Toxins.* Ph.D. Thesis, University of Hawaii, Honolulu.

TACHIBANA, K., P. J. SCHEUER, Y. TSUKITANI, H. KIKUCHI, D. VAN ENGEN, J. CLARDY, Y. GOPICHAND, AND F. J. SCHMITZ. 1981. Okadaic acid, a cytotoxic polyether from two marine sponges of the genus *Halichondria. J. Am. Chem. Soc.* **103:** 2469–2471.